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## Mechanisms for increased risk of diabetes in chronic liver diseases

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### Abstract

**Objective:** Patients with chronic liver disease (CLD), both non-alcoholic fatty liver disease (NAFLD) and chronic hepatitis C (CHC), are at high risk of diabetes (T2D), but mechanisms are still unknown. Muscle/liver insulin resistance (IR) and pancreatic dysfunction are the major metabolic defects leading to T2D. However, if the risk of T2D in CLD patients is because of reduced insulin response and/or to IR, and the impact of liver histology has not been investigated.

**Design:** We studied 220 non-T2D patients with chronic liver disease (129 NAFLD, BMI = 27.3 kg/m<sup>2</sup>; 91 CHC, BMI = 25.0 kg/m<sup>2</sup>) that received a 75-gram oral glucose tolerance test (OGTT) with the measurement of glucose and insulin concentrations for 2 hours, glucose tolerance (NGT vs IGT) and liver biopsy. The results were compared to 26 controls (CT-NGT, BMI = 25.6 kg/m<sup>2</sup>). We evaluated peripheral insulin sensitivity (OGIS), OGTT-insulin response ( $\Delta\text{AUC-I}/\Delta\text{AUC-G}$ ) and disposition-index ( $\text{DI} = \text{OGIS} \cdot \Delta\text{AUC-I}/\Delta\text{AUC-G}$ ) for the risk to develop T2D.

**Results:** NAFLD had increased muscle IR (associated to NASH, steatosis and fibrosis), higher than in CHC or CT-NGT (OGIS = 8.9 vs 11.3 and 10.5 mL/min kg,  $P < .0001$ ). In NAFLD, OGTT-insulin response ( $\Delta\text{AUC-I}/\Delta\text{AUC-G}$ ) was the highest while it was significantly decreased in CHC (2.2 vs 1.1 and 1.6, NAFLD vs. CHC and CT-NGT,  $P < .005$ ). The highest T2D risk (low DI) was observed in CHC-IGT (7.5), CHC-NGT (13.5) and NAFLD-IGT (10.8) vs CT-NGT (14.9, all  $P < .0001$ ), but not in NAFL-NGT or NASH-NGT.

**Conclusion:** We observed an increased T2D risk in NAFLD-IGT, CHC-IGT and CHC-NGT mainly because of reduced OGTT-insulin response, while insulin response in NAFLD-NGT compensates the IR thus maintaining normal glycaemia.

## INTRODUCTION

In 2019, the global prevalence of type 2 diabetes (T2D) in adults aged 20-79 years was estimated to be 9.3%, but more importantly, more than half (50.1%) of subjects with T2D did not know to have the disease.<sup>1</sup> Chronic liver disease (CLD) is a major risk factor for decreased glucose tolerance and development of type 2 diabetes (T2D).<sup>2-4</sup> Among CLDs, non-alcoholic fatty liver disease (NAFLD) is now recognized as an emerging metabolic disease, associated with increased T2D risk and affecting almost 25% of the world population.<sup>5</sup> Similarly, but to a less extent, exposure to hepatitis C virus (HCV) represents a well-known risk factor for T2D.<sup>6</sup> Many studies have shown that all CLDs are associated with insulin resistance (IR)<sup>7-9</sup> although obesity is often not accounted as a covariate despite being an independent risk factor for IR and T2D. Moreover the impact of reduced  $\beta$ -cell function and insulin secretion on the risk of T2D in CLD is usually not investigated.

In the pathophysiology of T2D, not only IR but also pancreatic  $\beta$ -cell function and insulin secretion play a major role.<sup>10-14</sup> As subjects become insulin resistant (mainly in the periphery), their glucose tolerance is maintained until the amount of insulin secreted by the pancreas is no longer sufficient to overcome the muscle IR.<sup>8, 10, 11, 14</sup> Both hepatic and pancreatic dysfunction are major defects that might explain the increased risk of T2D in subjects with liver disease. Alterations in hepatic glucose production and increased postprandial glycaemia are early signs of glucose intolerance<sup>14</sup> especially in patients with advanced liver disease even if they have normal fasting glucose concentrations (FPG).<sup>15</sup>

If and how IR and insulin secretion are altered in NAFLD and/or CHC patients is still unknown. The disposition index (DI, calculated from the insulin secretion factored by the insulin resistance) evaluates if the pancreatic insulin secretion is sufficient to overcome peripheral insulin resistance and maintain glycaemia within normal ranges.<sup>10, 16, 17</sup> Indeed, a low disposition index has been shown to be an independent marker of development of T2D.<sup>17-20</sup> However, to the best of our knowledge the disposition index has not been evaluated in patients with CLD diagnosed by liver biopsy. Moreover only few studies have investigated  $\beta$ -cell function and insulin response to a glucose load in subjects with NAFLD or CHC.<sup>15, 21-25</sup> Despite high hepatic fat accumulation and peripheral IR,  $\beta$ -cell function was often found unaltered in NAFLD<sup>21-23, 25</sup> indicating that most of the patients with CLD are able to compensate their IR by increasing insulin secretion and/or decreasing hepatic insulin clearance. Previously, Grancini et al have evaluated insulin secretion and  $\beta$ -cell function in 160 advanced cirrhotic patients candidates for liver transplantation (the great majority with HCV) that underwent OGTT finding that insulin secretion worsens with the worsening of liver disease, and transition from IGT to DM is driven primarily by  $\beta$ -cell dysfunction.<sup>15</sup> Narita et al have performed OGTT in patients with CHC but IR and  $\beta$ -cell function were evaluated only according to glucose tolerance and not in relation to the histological severity of the disease.<sup>24</sup>

Here we evaluated if and how the type of CLD (NAFLD vs CHC) and severity of liver disease (degree of liver fibrosis) are associated to reduced peripheral insulin sensitivity and insulin response during an OGTT and how the increased risk of T2D of subjects with liver disease (evaluated by the disposition index) is related to alterations of the pancreas-liver cross-talk.

## **METHODS**

### **2.1 Study subjects and protocol**

This is a post-hoc analysis of data of 220 non-diabetic patients with liver disease (NAFLD  $n = 129$ , CHC  $n = 91$ ) that participated to other protocols and that had an OGTT and liver biopsy.<sup>26, 27</sup> The protocol of data collection was part of the common clinical practice in the hospital units of University of Ancona and Torino. Clinical data have been already published<sup>26, 27</sup> while the analysis of  $\beta$ -cell function is completely new. All subjects were requested to give their informed consent to the use of personal data, analyses and liver biopsy at time of admission. CHC patients were recruited in Ancona between 2003 and 2005 and liver biopsy was performed to evaluate the degree of liver injury before the introduction of Fibroscan in the routine clinical management of these patients in order to define the degree of stage of liver injury and the need of antiviral treatment. CHC was defined by high transaminase values for more than 6 months and by the presence of serum HCV-RNA in the absence of coinfection with hepatitis B virus (hepatitis B surface antigen and core antibody-positive), autoimmune hepatitis, cholestatic (primary biliary cholangitis, primary sclerosing cholangitis) or genetic (haemochromatosis,  $\alpha$ 1-antitripsin deficiency, Wilson disease) liver disease. Subjects with pharmacologically treated diabetes or previous antiviral treatment were also excluded. The habitual alcohol intake in the last 6 months was assessed by interviews extended to family members and general practitioners, and patients with alcohol consumption  $> 40$  g/day were excluded. Other exclusion criteria were the presence of decompensated cirrhosis, the presence of HCC or non-hepatic neoplastic diseases or a low life expectancy because of comorbidities.

NAFLD patients were recruited in Torino and criteria for performing liver biopsy were previously reported,<sup>26, 27</sup> that is, chronically elevated aminotransferase levels (alanine aminotransferase (ALT) 1.5 times the upper normal limit for 6 months or more), negative hepatitis B (hepatitis B surface antigen and core antibody) and C (anti-HCV IgG) viral markers, absence of autoimmune hepatitis or coeliac disease, no evidence of genetic, drug-induced or cholestatic liver disease and alcohol consumption (ie less than 20 g/d). Moreover 26 healthy subjects ( $BMI = 25.6 \text{ kg/m}^2$ ) previously tested with normal glucose tolerance (CT-NGT), without liver disease by ultrasound or, when not available, by fatty liver index, were used as control group.

Liver biopsies were available in all CHC and NAFLD patients and were scored in a blinded manner by two pathologists at the University of Ancona (for CHC patients) and at the University of Turin (for NAFLD patients). Fibrosis was scored according to Metavir<sup>28</sup> for CHC and Kleiner score for NAFLD.<sup>29</sup> NASH was diagnosed by the joint presence of steatosis, ballooning and lobular inflammation.<sup>30</sup> Patients with a fibrosis score  $\geq$  F2 were defined as high fibrosis (HF).

All subjects received an oral glucose tolerance test (OGTT, 75g) with analysis of the glucose and insulin profiles at 0, 30, 60, 90, and 120 minutes after glucose ingestion. Data in CHC and NAFLD patients were compared with those obtained in CT-NGT subjects that were divided according to their response to OGTT as insulin sensitive (NGT-IS) if  $\text{OGIS} > 9.8 \text{ mL/kg/min}$ , vs insulin resistant (NGT-IR).<sup>26</sup>

This study was approved by the Ethical Committees of the participating centers (550-586-70-2009 for University of Turin and 205 731 for University of Ancona), regulating non-interventional studies. Glucose concentrations were measured with an automated analyser (Beckman Instruments, Fullerton CA, USA; inter-assay coefficient of variation (CV) 4%) while insulin concentrations were measured by immune-histochemistry assay (AIA-PACK IRI, AIA-1200 system, Tosoh Co.) with intra- and inter-assay CVs for quality control  $< 7\%$  as described previously.<sup>26, 27</sup>

## 2.2 Calculations

Subjects were analysed utilizing the following categories: control subjects were divided according to their response to OGTT as insulin sensitive (NGT-IS) if  $\text{OGIS} > 9.8 \text{ mL/kg/min}$ , vs insulin resistant (NGT-IR); CHC patients were divided according to genotype (G3 vs non-G3) or according to glucose tolerance (CHC-NGT vs CHC-IGT); NAFLD patients were further categorized as NAFL or NASH, or according to glucose tolerance (NAFL-NGT, NASH-NGT, NAFL-IGT, NASH-IGT). Patients were also analysed according to fibrosis score, that is, low (LF) fibrosis (F0-F1) vs high (HF) fibrosis (F2-F4). NAS score was calculated as the sum of scores for steatosis, lobular inflammation and ballooning.<sup>30</sup>

Peripheral insulin sensitivity was assessed during OGTT by oral glucose insulin sensitivity (OGIS) index<sup>31</sup> and at fasting by HOMA.<sup>32, 33</sup>

Insulin response to OGTT was assessed by calculating the ratio of incremental area under the curve (AUC) of insulin to glucose  $\Delta\text{AUC-I}/\Delta\text{AUC-G}$  from 0-120 minutes.<sup>10</sup> The rapid insulin response was assessed as  $\Delta\text{AUC-I}/\Delta\text{AUC-G}$  from 0-30 minutes (Insulinogenic index, IGI).<sup>34</sup>

As previously stated, when subjects become insulin resistant (IR), normal glucose tolerance is maintained until amounts of insulin secreted by the pancreas are sufficient to overcome the muscle IR.<sup>8, 10, 11</sup> The relationship between insulin sensitivity and insulin secretion is hyperbolic as shown by Bergman et al<sup>35</sup> Thus, by measuring the insulin secretion factored insulin resistance (the so-called disposition index,  $DI = OGIS \times \Delta AUC-I / \Delta AUC-G$ ) it is possible to evaluate if the pancreatic insulin secretion is sufficient to overcome peripheral insulin resistance and maintain glycaemia within normal ranges.<sup>10</sup>

## 2.3 Statistical analysis

For statistical analysis patients were grouped as NAFLD ( $n = 129$ ,  $BMI = 27.3 \text{ kg/m}^2$ ), CHC genotype 3 (G3,  $n = 20$ ,  $BMI = 24.1 \text{ kg/m}^2$ ) or CHC non-3 genotype (non-G3,  $n = 71$ ,  $BMI = 25.2 \text{ kg/m}^2$ ) since CHC genotype 3 is known to have a different metabolic profile from CHC non-3 genotype.<sup>7</sup> CT subjects were grouped as insulin sensitive (NGT-IS) or insulin resistant (NGT-IR) according to their response to OGTT (ie NGT-IR if Oral Glucose Insulin Sensitivity index  $OGIS \leq 9.8 \text{ ml/min kg}$ )<sup>26, 27</sup> to better identify differences because of liver disease vs IR.

Data are given as the mean  $\pm$  SE. Group differences were analysed by Student *t* test, Mann-Whitney test, and chi-squared test, for normally distributed, non-normally distributed and non-continuous variables respectively. Univariate analysis (Spearman correlation coefficient) was used to estimate associations among continuous variables in the whole dataset. A two-tailed  $P < .05$  was considered statistically significant. The correlation coefficient and p-values are reported in the graphs as well as in the text.

## RESULTS

### 3.1 Clinical characteristics of the study subjects

We studied 220 non-diabetic patients with liver disease (NAFLD  $n = 129$ ; CHC  $n = 20$  for G3 and  $n = 71$  for non-G3) and compared to 29 controls without liver disease (Table 1). Control subjects were further divided into two groups as insulin sensitive (NGT-IS) or insulin resistant (NGT-IR), according to  $OGIS \leq 9.8 \text{ mL/min kg}$  (Table 1).

CHC patients had a mean  $BMI = 25.0 \pm 0.4 \text{ kg/m}^2$  ( $BMI = 24.1 \pm 0.7$  and  $25.2 \pm 0.5 \text{ kg/m}^2$  for G3 and non-G3 respectively) not different from NGT-IS subjects ( $BMI = 25.9 \pm 0.8 \text{ kg/m}^2$ ) while NAFLD patients ( $BMI = 27.3 \pm 0.3 \text{ kg/m}^2$ ) had a BMI slightly higher than CHC but not controls (Table 1).

AST and ALT were significantly increased in CHC and NAFLD compared to NGT-IS, while GGT was increased only in NAFLD and CHC non-G3 (Table 1).

Lipid profile was similar among the groups, although CHC tended to have lower total cholesterol concentrations. The highest triglyceride (TG) concentrations were observed in the NAFLD group as expected, and in CHC non-G3 (Table 1).

### **Glucose tolerance in NAFLD vs CHC patients**

All patients underwent a standard OGTT with the measurement of glucose and insulin levels every 30 minutes for 2 hours to assess glucose tolerance (Figure 1). The great majority of the patients had normal glucose tolerance (74% of patients were NGT, Table 1). Based on OGIS index, control subjects were divided into insulin sensitive (NGT-IS), if  $OGIS > 9.8 \text{ mL/kg/min}$ , vs insulin resistant (NGT-IR). Fasting glucose concentrations were lower in CHC compared to NAFLD, and in CHC-G3 they were lower than in controls (Table 1). The great majority of the patients had normal fasting glucose concentrations (80% were NFG, ie  $<100 \text{ mg/dL}$ ) and a similar distribution was observed in both CHC and NAFLD (impaired fasting glucose, IFG, in 14% and 24% respectively, Table 1).

The highest prevalence of impaired glucose tolerant (IGT) subjects was observed in CHC non-G3 (31%), while in NAFLD it was similar to CHC G3 and control subjects with insulin resistance (26%, 25% and 25% respectively, Table 1). A higher prevalence of IGT was observed also in NAFLD with F2-F4 (Table 2).

### **Insulin sensitivity in NAFLD vs CHC patients**

Fasting insulin resistance was measured by HOMA that was increased only in NAFLD compared to CT-NGT. In CHC patients, HOMA was significantly lower than in NAFLD while there was no difference with CT-NGT despite a lower mean value (Table 1).

Peripheral insulin sensitivity during OGTT was evaluated by OGIS index that is a surrogate measure of glucose clearance during OGTT.<sup>27, 31</sup> In average, NAFLD had the lowest OGIS ( $8.9 \text{ mL/min kg}$  vs  $11.3 \text{ mL/min kg}$  in CHC and  $10.5$  in NGT-IS  $\text{mL/min kg}$ ,  $P < .0001$ ). However, nearly half of the subjects with NAFLD ( $n = 55$ ) had an OGIS index  $> 9.8 \text{ mL/min kg}$ . On the contrary, subjects with CHC non-G3 had an OGIS similar to NGT-IS while CHC G3 were even more sensitive than NGT-IS (Table 1).

When the analysis was performed only in non-obese subjects (ie  $BMI \leq 30$ ), that comprised the great majority of patients (85%, ie 167/200), the results were similar.

In NAFLD, OGIS was negatively correlated to liver fat in biopsy ( $r = -0.31$ ,  $P = .0005$ ) while no correlation was found in patients with CHC. However, the prevalence of hepatic steatosis was extremely low in patients with CHC and non G3 genotype, while it was higher in the G3 genotype that has a viral pathogenesis (Table 1).

Since a decrease in OGIS was previously associated to increased liver fibrosis in NAFLD,<sup>27</sup> we also evaluated if the degree of liver fibrosis had an impact on either glucose clearance or insulin response (Table 2, Figure 2). In the entire cohort of subjects with liver disease, patients with advanced fibrosis (F3-F4) had decreased OGIS but, when evaluated separately, only in NAFLD, and not in CHC, reduced OGIS was associated to increased liver fibrosis and this remained significant also after adjusting for BMI and gender (partial  $r = -0.19$ ,  $P = .02$ ), but correlation was lost if further adjusted for age (partial  $r = -0.15$ ,  $P = .08$ ) (Figure 2 panel C). A NAS score greater than 3 (Figure 2 panel E) or the presence of NASH were both associated with significantly reduced OGIS.

These data indicate that, although not diabetic, NAFLD patients show decreased peripheral insulin sensitivity compared to controls and this decrease is associated with a higher degree of liver injury. No modifications have been observed in either G3 and non-G3 CHC patients compared to controls.

### **Insulin response to OGTT in NAFLD vs CHC patients**

Insulin response to OGTT was assessed by calculating the  $\Delta\text{AUC-I}/\Delta\text{AUC-G}$  from 0 to 120 minutes (Figure 2 panel B). The highest fasting and OGTT insulin concentrations were observed in NAFLD (Figure 1, panel B) despite glucose concentrations were similar to NGT-IR and CHC, indicating a preserved capacity to maintain glucose tolerance by adjusting insulin secretion/hepatic insulin clearance.

In subjects with NAFLD, the insulin response to increased glucose concentrations after OGTT was much higher than in CT-NGT while  $\Delta\text{AUC-I}/\Delta\text{AUC-G}$  was significantly lower in CHC compared to CT-NGT and NAFLD (1.1 vs 2.2 and 1.6, CHC vs NAFLD and NGT-IS,  $P < .005$ ) (Figure 1, panel B).

In CHC G3 patients, fasting insulin concentrations were lower than in CT-NGT (Table 1) while during OGTT they increased as high as NGT-IR. However, CHC patients, especially those non-G3, had a reduced response in the first 30min, indicating a loss of first phase insulin secretion. In NAFLD, the insulinogenic (IGI) index that reflects the early insulin response was similar to NGT-IS (Table 1) while  $\Delta\text{AUC-I}/\Delta\text{AUC-G}$  was even higher than in NGT-IS (Figure 2 Panel B).

In NAFLD, but not in CHC,  $\Delta\text{AUC-I}/\Delta\text{AUC-G}$  was negatively correlated to liver fat in biopsy ( $r = 0.20$ ,  $P = .03$ ).

No significant association was found between insulin response ( $\Delta\text{AUC-I}/\Delta\text{AUC-G}$ ) and the degree of liver fibrosis in either CHC or NAFLD (Table 2 and Figure 2 panel D), nor with NAS score (Figure 2 panel F) or presence of NASH.



Taken together, these data indicate that the insulin levels after OGTT are increased in NAFLD patients to maintain glucose concentrations during OGTT within normal limits, while CHC patients have an insulin profile comparable to NGT-IR.

### **Disposition index (DI) in NAFLD vs CHC**

In the above analyses, we have shown that patients with NAFLD were able to increase insulin response to overcome reduced insulin sensitivity and thus maintain glucose tolerance and do not develop hyperglycaemia and T2D.

In each group, we evaluated the disposition index (DI calculated as the product of insulin response times insulin resistance sensitivity) according to glucose tolerance status (ie normal glucose tolerance, NGT, vs impaired glucose tolerance, IGT). DI is an index of pancreatic insulin response factored by insulin resistance and inversely associated to increased risk of  $\beta$ -cell dysfunction and T2D.<sup>18, 19, 36</sup> In Panel A of Figure 3 lines are the trajectories of insulin sensitivity and secretion for each given DI and explain why until the subjects remain on the same line they are maintaining a normal glucose tolerance (NGT), that is, the increased insulin response allows to compensate the reduced insulin sensitivity, and DI is preserved. NGT patients with NAFLD have the same DI, that is, similar risk to develop T2D, since NAFL-NGT, NASH-NGT and NGT-IS are on similar curve (ie similar DI =  $24.6 \pm 3.0$ ,  $18.2 \pm 1.5$  vs  $18.0 \pm 3.1$ , respectively,  $p = ns$ ), despite different IR and insulin secretion (Figure 3, Panel B).

DI was significantly decreased in CHC, particularly if IGT, but not in this group of NAFLD ( $11.8 \pm$  vs  $18.2 \pm$  vs  $16.8 \pm$  in CHC vs NAFLD vs NGT-IS,  $P < .0001$ ) confirming the above observation (Figure 4, Panel A).

DI was not associated to the degree of steatosis, neither in NAFLD, nor in CHC and neither the degree of fibrosis was associated with DI (Figure 4 panel B), but DI was instead decreased with increased glucose intolerance in both NAFLD and CHC (Figure 3 panel B). NAFLD patients with increased NAS score and/or the presence of NASH had lower DI (Figure 4 panel C and D).

### **DISCUSSION**

In this study, we evaluated data from 220 non-diabetic patients (NAFLD  $n = 129$  and CHC  $n = 91$ ) with liver biopsy to verify: (a) if and how the type (NAFLD vs CHC) and severity of liver disease (grade of liver inflammation and stage of fibrosis, and presence of NASH) are associated to reduced peripheral insulin sensitivity and/or reduced insulin response during an OGTT; (b) how the increased risk of T2D in subjects with liver disease (evaluated by the DI) is related to alterations in the pancreas-liver cross-talk. The results of this analysis indicate that, although not diabetic, NAFLD patients had decreased peripheral insulin

sensitivity compared to controls and this decrease was associated with presence of NASH and a higher degree of liver injury, while insulin sensitivity of CHC patients was similar to controls. Insulin response to oral glucose load was increased in NAFLD patients compared to both NGT-IS and NGT-IR, but compatible with the degree of IR, so that glucose concentrations during OGTT were within normal limits. On the contrary, CHC patients had a reduced insulin profile comparable to NGT-IR. Considering the risk of T2D (indicated by a low DI) in CLD, the higher risk was observed in CHC and was not associated with the stage of fibrosis, but to the degree of glucose tolerance. This poses the attention on patients that were previously exposed to HCV since this might have altered  $\beta$ -cell function thereby favouring the pathogenesis of T2D.<sup>37</sup>

Epidemiological studies have shown that both NAFLD and CHC are major risk factors for decreased glucose tolerance and development of type 2 diabetes (T2D).<sup>2-4, 38</sup> The prevalence of both diabetes and NAFLD is increasing<sup>1, 5</sup> and it has been estimated that almost half of the subjects with diabetes do not know to have the disease since they still have normal fasting glucose concentrations.<sup>1</sup> It is recognized that postprandial hyperglycaemia occurs much earlier than fasting glycaemia<sup>13</sup> since the pancreatic insulin secretion in response to changes in glucose concentrations (eg after a meal) is often not sufficient to overcome the peripheral insulin resistance state, thus determining high postprandial glucose levels. Abnormalities in glucose tolerance occur frequently in chronic liver diseases,<sup>14</sup> not only in the advanced state and even in patients with normal FPG,<sup>15</sup> supporting the importance of performing oral glucose tolerance tests (OGTT). In the pathophysiology of T2D, IR and in particular impaired insulin secretion and  $\beta$ -cell dysfunction are important risk factors, but if they are both altered in NAFLD and CHC patients is still not clear.

The OGTT, with the simultaneous measurement of glucose and insulin concentrations, serves not only to assess glucose tolerance but also insulin secretion,<sup>10</sup> insulin resistance in the muscle and liver<sup>27, 31</sup> and increased risk to develop type 2 diabetes.<sup>10, 19, 36</sup> Peripheral and hepatic IR are characteristic features of patients with CHC<sup>7</sup> or NAFLD,<sup>14</sup> even if they are lean.<sup>39</sup> In our recent paper in non-diabetic NAFLD,<sup>27</sup> we have shown that, in non-diabetic NAFLD, glucose concentrations during OGTT were similar in obese and non-obese patients independent of the degree of liver steatosis, while fibrosis score F2-F4 was associated with increased glucose concentrations during OGTT independent of obesity. On the contrary, insulin concentrations were lower only in non-obese with low steatosis and fibrosis.

We have found that insulin response during OGTT was higher in patients with NAFLD compared to the other groups (Figure 1) in line with other studies<sup>23, 27</sup> although appropriate for their glucose tolerance status.<sup>14</sup> On the contrary, in patients with CHC we have found

that insulin sensitivity was similar to insulin sensitive of CT-NGT, while glucose-stimulated insulin release was reduced compared to both NAFLD and CT-NGT (Figure 2). Although HCV replicates principally in hepatocytes also other organs might be affected by the virus, like muscle and pancreas.<sup>7, 37, 40</sup> This might explain the alterations in glucose metabolism and tolerance and insulin resistance often observed in CHC patients and their high risk to develop T2D.<sup>7, 15</sup> Masini et al have analysed the pancreatic islets of patients with CHC<sup>37</sup> finding that these patients have both morphological and functional defects, in particular reduced glucose-stimulated insulin release that agrees with our findings (Figure 2, panel B). It has been shown that peripheral insulin sensitivity and glucose metabolism parameters ameliorate after HCV eradication,<sup>41-43</sup> but if  $\beta$ -cell dysfunction improves after Directly Acting Antivirals, or if morphological and functional defects because of virus exposure are permanent, will need further investigation.

The risk to develop T2D was assessed by the evaluation of the insulin secretion/insulin resistance relationship (also named disposition index, DI).<sup>10, 17</sup> The disposition index follows a hyperbolic curve that indicates that, until the pancreatic insulin response compensates the reduced insulin sensitivity (see arrows in Figure 4), the subjects preserve their insulin tolerance status, while a decrease in DI indicates an increased risk of T2D.<sup>20, 35</sup> With the decrease in DI, the patients move on a lower curve increasing their risk to become T2D (ie low DI given by a low insulin secretion and/or high insulin resistance).

In this cohort, DI was significantly reduced in CHC but not in NAFLD (Figure 4). This was mainly because of the decreased insulin response during OGTT. On the contrary, NAFLD is on the same line as CT despite high insulin resistance (Figure 4). Thus, in this cohort of patients with NAFLD the insulin response is more than adequate to overcome the defect in peripheral insulin resistance thus maintaining glucose tolerance. This is probably because of the fact that most of these patients are non-obese. However, we cannot exclude that the high insulin response in the long term might result in  $\beta$ -cell stress and dysfunction, thus predisposing to diabetes.

We observed that subjects with increased liver fibrosis, in particular NAFLD patients, had lower DI due in part to decreased peripheral insulin sensitivity (OGIS index) but also to peripheral insulin concentrations. We cannot establish if these differences were because of pre-hepatic insulin secretion rates or hepatic insulin clearance since we did not measure C-peptide concentrations. In normal conditions, the liver clears up to 60% of the secreted insulin during the first pass, while it does not degrade C-peptide.<sup>44</sup> In liver disease, and/or IR, insulin clearance is reduced in order to have higher insulin concentrations in the periphery.<sup>21, 45-47</sup> Thus, we cannot establish if increased insulin concentration during OGTT is the result of increased insulin secretion or reduced hepatic clearance or both. However, a recent article has shown that despite NAFLD had a reduced insulin clearance this was

dependent on reduced peripheral insulin sensitivity and subcutaneous fat, rather than the degree of liver steatosis.<sup>48</sup> Moreover this is not a limit for the calculation of DI since, as we have shown recently, only peripheral insulin concentrations and not C-peptide are related to insulin sensitivity in a hyperbolic manner and thus insulin and not C-peptide should be used to calculate DI.<sup>20</sup>

Another possible limitation is the inclusion in this analysis of mainly non-obese NAFLD patients in order to have a good match for BMI with the CHC cohort. Obesity is often associated to IR and alteration in  $\beta$ -cell function. However, even in morbid obese subjects, glucose tolerance is often preserved and presence of NAFLD is not associated to impairment in glucose stimulated insulin response.<sup>49</sup> Moreover, parameters of  $\beta$ -cell function such as glucose sensitivity (ie dose-response insulin secretion-glucose concentration), first phase insulin secretion, and potentiation, do not appear to be substantially altered by obesity as long as glucose tolerance is maintained.<sup>50</sup>

The degree of fibrosis was associated to a reduced OGIS only in NAFLD patients, and it was not associated with DI. On the contrary, DI was decreased with worsening of glucose intolerance in both NAFLD and CHC ( $r = -0.48$ ,  $P < .0001$ ). Why OGIS was associated to hepatic fibrosis only in patients with NAFLD is controversial. The most plausible explanation is that insulin resistance (IR) is the main driver of the hepatic pathological events that finally lead to liver fibrosis, and insulin per se exerts a direct fibrogenetic effect on hepatic stellate cells (HSCs).<sup>51, 52</sup> As a confirmation, reduced OGIS was also observed in NAFLD patients with the higher NAS score (steatosis, lobular inflammation and ballooning) that can predispose to fibrosis. On the contrary, although diabetes has been reported to affect almost 15% of HCV patients,<sup>38</sup> the pathogenesis of HCV-induced liver injury is mostly based on a series of virus-associated events.<sup>53</sup> HCV proteins modulate hepatocyte apoptosis and necrosis leading to HSCs and fibrosis. In addition, HCV manipulates the immune system that initially attempts to eradicate the virus, but, in the setting of chronic infection, promotes hepatocyte damage and fibrosis through direct cellular toxicity and the release of inflammatory cytokines. Thus, we can speculate that the role of IR is of minor importance in determining the degree of liver fibrosis in CHC, and this is why liver fibrosis is associated with OGIS in NAFLD and not in CHC.

In conclusion, the liver plays a central role in metabolic disturbances. The presence of both hepatic and pancreatic dysfunction are major defects that explain the increased risk of T2D in patients with liver disease. Although decreased OGIS was associated to the degree of steatosis, fibrosis and NAS score, patients with NAFLD are able to compensate the increased muscle IR modulating peripheral insulin concentrations. On the contrary, most of CHC patients have an impairment in insulin response to OGTT that increases their risk to develop T2D. While diagnosis of advanced fibrosis by non-invasive tests and treatment is

well-defined in HCV patients, these aspects are challenging in NAFLD patients. OGIS measurement after OGTT should be performed in routine clinical practice to identify those NAFLD patients at risk of T2DM and with fibrosis.

**TABLE 1.** Clinical characteristics of study subjects

- Abbreviations: IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NFG, normal fasting glucose; NGT, normal glucose tolerance; NGT-IR, control normal glucose tolerant – insulin resistant; NGT-IS, controls normal glucose tolerant – insulin sensitive.
- <sup>a</sup> Presence of steatosis at liver biopsy.
- <sup>b</sup>  $P < 0.05$  vs NGT-IS.
- <sup>c</sup>  $P < 0.05$  vs NGT-IR.
- <sup>d</sup>  $P < 0.05$  vs NAFLD.
- <sup>e</sup>  $P < 0.05$  vs CHC non-G3.

**TABLE 2.** Clinical and metabolic characteristics of subjects with liver disease according to the degree of fibrosis

- <sup>a</sup>  $P < .05$  F2-F4 vs F0-F1.

**FIGURE 1**

Glucose (Panel A) and Insulin (Panel B) profile during a standard OGTT (75 g) in controls (insulin sensitive, NGT-IS and insulin resistant, NGT-IR), CHC and NAFLD. Statistical analysis  $P < .05$ : \* vs NGT-IS, <sup>§</sup> vs NGT-IR, # vs CHC, † vs NAFLD

**FIGURE 2**

Insulin sensitivity (OGIS index) and insulin secretion calculated as insulin response to OGTT in controls NGT-IS and NGT-IR, CHC and NAFLD (panel A and B); in CHC and NAFLD according to the degree of fibrosis (panel C and D); in NAFLD according to NAS score (panel E and F). The degree of fibrosis was increased with lower OGIS independent of BMI and with increased glucose intolerance in both NAFLD and CHC ( $r = -0.48$ ,  $P < .0001$ ). Statistical analysis  $P < .05$ : \* vs NGT-IS, <sup>§</sup> vs NGT-IR, # vs NAFLD; † vs low Fibrosis; ‡ vs NAS 1-3

**FIGURE 3**

Panel A. Disposition index (DI) trajectories in subjects with or without liver disease. A low DI is a sign of increased risk of type 2 diabetes. In this cohorts, insulin sensitive control subjects (NGT-IS), NAFL-NGT and NASH-NGT were on similar curves, indicating that NAFLD patients compensated the lower IS with increased insulin response during OGTT. Subjects with CHC-NGT have reduced DI, similar to NGT-IR, NAFL-IGT and NASH-IGT, while in CHC-IGT the DI was further reduced. Panel B shows the mean values of DI in each group (\* $P < .05$  vs NGT-IS, <sup>§</sup> $P < .05$  vs NGT-IR, † $P < .05$  vs CHC-

NGT, <sup>#</sup>*P* < .05 vs NAFL-NGT <sup>§</sup>*P* < .05 vs NASH-NGT following Mann-Whitney comparison among groups)

#### FIGURE 4

The disposition index (DI) in controls (NGT-IS and NGT-IR), CHC and NAFLD (panel A); in CHC and NAFLD according to the degree of fibrosis (panel B); in NAFLD according to NAS score (panel C); in NAFL vs NASH score (panel D). The degree of fibrosis was not associated with DI. \**P* < .05 vs NGT-IS, <sup>§</sup>*P* < .05 vs NGT-IR, <sup>#</sup>*P* < .05 vs NAFLD; <sup>†</sup>*P* < .05 vs low NAS 1-3

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